DESCRIPTION

Plant Th rmogenic Genes and Proteins

Technical Field

The present invention relates to plant thermogenic genes and proteins. More particularly, the invention relates to thermpogenic genes derived from a skunk cabbage (*Symplocarpus foetidus*) and gene products (proteins). - Those genes and proteins are useful in breeding of cold-avoidance plants, medical treatment of diabetes mellitus or obesity, or development of novel thermogenic bio-materials.

Background

Stresses due to low temperatures, droughts and salinity are common harmful environmental factors that terrestrial plants encounter. Among these stresses, it has been considered that cellular injury due to the low temperature is the most important factor which restricts productivity of crops (Levitt, 1980). To resist the low temperature stress, cold-hardy plants such as wheat or rye have a variety of physiological and metabolic responses which lead to cold acclimation (Sakai and Larcher, 1987; Steponkus, 1984; Thomashow, 1998; Uemura and Steponkus, 1997). In contrast, it is known that some plants including skunk cabbage have a specialized system by which the plants generate heat to avoid freezing (Knutson, 1974; Nagy et al., 1972; Schneider and Buchenen, 1980).

The temperature of the flower in the spadix of skunk cabbage, which flowers in early spring, has been known to maintain its temperature at higher

than +10°C even when the ambient temperature falls to -15°C (Knutson, 1974). For example, thermoscopic analysis using infrared cam ra indicates homeothermic behavior of the surface temperature f the spadix (Fig. 1). It should be noted that, in this experiment, the plants were placed in the growth chamber and the air temperature was gradually decreased. As clearly seen from Fig. 1, the temperature of the spadix of skunk cabbage is kept at approximately 19°C notwithstanding a fall of the ambient temperature.

The temperature is thus maintained by doubling the respiration rate from the level of 12°C to that of sub-zero temperature. It has also been considered that the heat production in thermogenic plant species relates to a cellular metabolism called cyanide-non-sensitive/non-phosphorylating electron-transferring pathway, which is controlled by mitochondrial alternative oxidase (AOX)(Berthold and Siedow, 1993; Ito et al., 1997; McIntosh, 1994, Wangner and Krab, 1995).

On the other hand, it has been shown that a mitochondrial protein called an uncoupling protein (UCP) plays an important role in generation of heat in mammals. UCP found in the intima of mitochondria make H^+ flow into the membrane to uncouple aspiration from synthesis of ATP which acts to disperse chemical energy to metabolic heat (Klaus et al., 1991; Klingenberg and Winkler, 1985; Ricquier et al., 1991). In animals, 3 types of UCPs have been found. UCP1 is primarily distributed in brown adipose tissue (Nicholllus and Locke, 1984). UCP2 is found ubiquitously in many tissues (Fleury et al., 1997), and UCP3 is localized specifically in skeletal muscle (Boss et al., 1997).

It has been considered that UCPs of mammals, similarly to other carrier proteins of mitochondria, are composed of 6 transmembrane segments, of which the hydrophobic portion is derived from pairing amphipathic α-helix structure (Liu et al., 1988; Maia et al., 1998). It is also known that the activity of these UCPs decreases depending on purine nucleotides (ATP, GTP, GDP and ADP) attached to

the C-terminal region and increases by free fatty acids (Jezek et al., 1998; Lin and Klingenberg, 1982; Katiyar and Shrago, 1989; Rial et al., 1983; Sluse et al., 1998).

On the contrary, 2 cDNAs encoding UCP-like proteins of plant origin were isolated from potato (StUCP: Laloi et al., 1997) and from *Arabidopsis* (AtPUMP: Maia et al., 1998). Since the expression of StUCP was mainly detected in the flower and the fruit, it has been postulated that StUCP may concern respiration during flowering and maturation of the fruit together with the AOX activity (Laloi et al., 1997).

Potato and *Arabidopsis* have been considered to be non-thermogenic plants. However, the expression of StUCP and AtPUMP induced by low temperature. Therefore, it has been suggested that these genes are involved in the heat production (Laloi et al., 1997; Maia et al., 1998).

In the thermogenic plants such as skunk cabbage, however, UCP-mediated thermoigenic mechanisms have not yet been identified.

The purpose of the invention of this application is to provide unidentified novel UCP genes derived from a thermogenic plant, skunk cabbage.

The additional purpose of this application is to provide skunk cabbage UCPs which are expression products of the novel genes.

Disclosure of Invention

The invention provides thermogenic genes derived from skunk cabbage, i.e., gene SfUCPa of which cDNA comprises the base sequence of SEQ ID NO: 1, and gene SfUCPb of which cDNA comprises the base sequence of SEQ ID NO: 3.

Moreover, the invention provides thermogenic proteins, i.e., protein SfUCPA expressed from SfUCPa, which comprises the amino acid sequence of SEQ ID NO: 2, and protein SfUCPB expressed from SfUCPb, which comprises the amino acid sequence of SEQ ID NO: 4.

In addition, the invention provides cDNA having the base sequence of SEQ ID NO: 1 or a partial sequence thereof, and cDNA having the base sequence of SEQ ID NO: 3 or a partial sequence thereof.

Brief Description of Drawings

Fig. 1 shows the change of the temperature of the spadix in skunk cabbage and that of ambient temperature with a lapse of time.

Fig. 2 shows the results of northern blotting, indicating the expression profile of SfUCPa (A) and SfUCPb (B) in the spadix and leaf of skunk cabbage at room temperature (RT) and during cold treatment (4°C for 3 days). The lower figures respectively show the results of ethidium bromide staining of non-decomposed rRNA.

Fig. 3 compares the alignment of amino acid sequences of SfUCPA and SfUCPB, together with potato UCP (StUCP), *Arabidopsis* UCP (AtPUMP) and human UCP. The asterisks (*) attached under the sequences indicate the same amino acid sequence, and the dot (.) indicates the conservative change in all of the sequences. The boldface indicates the same sequence between SfUCPA and SfUCPB. The gap introduced to optimize the sequence alignment is indicated by a dash (-). The alignment was made using a CLUSTAL W program. The characteristic domains of energy transfer proteins typical of mitochondria are

surrounded by a square. The shaded bars (I-VI) above the upper sequence show estimated transmembrane domains.

Fig. 4 shows a hydrophobic plot of SfUCPA. The vertical axis indicates the degree of hydrophobicity and the estimated transmembrane domains are indicated by TM1 to TM6.

Fig. 5 shows a diagrammatic illustration of SfUCPA topology in the mitochondria membrane.

Fig. 6 shows a hydrophobic plot of SfUCPB. The vertical axis indicates the degree of hydrophobicity and the estimated transmembrane domains are indicated by TM1 to TM4 and TM6.

Fig. 7 shows a diagrammatic illustration of SfUCPB topology in the mitochondria membrane.

Fig. 8 shows the results of *in vitro* translation using respective cDNAs of the genes SfUCPa and SfUCPb as templates. (-) indicates a control, S a sense RNA, and AS an antisense RNA. The asterisk (*) indicates a non-specific product and the empty circle denotes the position of a low molecular translated artificial product synthesized from a small ORF.

Best Mode for Carrying Out the Invention

In the gene SfUCPa of the present invention, its cDNA has the base sequence of SEQ ID NO: 1 and encodes the protein SfUCPA having the amino acid sequence of SEQ ID NO: 2, of which the estimated molecular weight is 32.6 kDa. In the gene SfUCPb of the present invention, its cDNA (SEQ ID NO: 3) encodes

the protein SfUCPB having the amino acid sequence f SEQ ID NO: 4, of which the estimated molecular weight is 29.0 kDa.

Genes SfUCPa and SfUCPb of the invention are derived from skunk cabbage, which are expressed specifically in the spadix when the temperature is low. The results of Northern blotting on the total RNAs extracted from skunk cabbage (Ito et al., 1999), confirmed that the expressions of both genes were detected in the spadices but not in the leaves at room temperature (15°C) (Fig. 2). It was also confirmed that the spadix-specific expression of both genes were induced by cold treatment (4°C for 3 days).

The amino acid sequences of the proteins SfUCPA and SfUCPB that are expressed from the respective genes of the invention have higher homology to the plant UCPs than to the human UCPs (Fig. 3) such that the amino acid sequence of SfUCPA has homology of 79%, 75%, 44%, 48% and 48% to StUCP, AtPUMP, human UCP, UCP2 and UCP3, respectively. SfUCPB has homology of 71%, 66%, 41%, 43% and 44% to StUCP, AtPUMP, human UCP, UCP2 and UCP3, respectively.

In addition, SfUCPA and SfUCPB have high sequence homology (88%) to each other though the region corresponding to the amino acid sequence between the 204th Thr and the 238th Val in SfUCPA is completely deleted in SfUCPB (Fig. 3). Moreover, the 265th Leu of SfUCPA is replaced by Pro in SfUCPB.

StUCPA has similar structure to that of other mitochondria UCP proteins. SfUCPA has 6 transmembrane domains as shown by the hydrophobic plot in Fig. 4, of which the topology is as shown in Fig. 5. In addition, this SfUCPA has 3 domains that are characteristic of energy transfer proteins in mitochondria (Fig. 3)(Boss et al., 1997; Maia et al., 1998). On the other hand, SfUCPB is lacking in the 3rd domain which is characteristic of energy transfer proteins in mitochondrial

(Fig. 3), as well as in the 5th transmembrane domain (Figs. 3 and 6). The topology is located toward the mitochondria matrix at the C-terminal (Fig. 7).

Each protein has a purine nucleotide-binding domain (PNBD) at the C-terminal (Figs. 3, 5 and 7), and it is known that in UCP, binding of the purine nucleotide inhibits the uncoupling function in the mitochondria intima. In SfUCPB, however, there is a possibility that it may have escaped the inhibition of the binding of the purine nucleotide because its C-terminal is located toward the mitochondria matrix. Such a topology has not been found in any UCPs from animals or plants.

The thermogenic genes SfUCPa and SfUCPb provided by the invention are derived from skunk cabbage and are very useful in, for example, development of low temperature-tolerant plants using a genetic recombination technique. The proteins SfUCPA and SfUCPB that are expression products from the above genes are expected as effective components in remedies of diabetes mellitus, obesty, and the like, based on the uncoupling function to ATP synthesis. Moreover, such thermogenic proteins are also promising novel heat generating bio-materials.

The genes SfUCPa and SfUCPb of the invention can be isolated from the genomic DNA of skunk cabbage using the cDNA (SEQ ID NOS: 1 or 3) or a partial sequence thereof of the invention as a probe. For example, a genome library is prepared from the genomic DNA according to a known method. It may be screened by means of colony or plaque hybridization according to a known method using as a probe an oligonucleotide synthesized based on the base sequence of an optional portion of cDNA. Alternatively, the target genetic region may also be identified by means of in situ hybridization for chromosome.

The respective cDNAs of the invention can be cloned, for example, from a cDNA library which is synthesized using a poly(A)$^+$RNA of skunk cabbage as a

template. In such a case, an oligonucleotide of an optional portion of cDNA provided by th invention is synthesized, which may be used as a probe to carry out creening by mean of colony r plaque hybridization according to a known method. Alternatively, oligonucleotides which can hybridize to both ends of the target cDNA fragment are synthesized, which may be used as primers in preparation of cDNA of the invention by the RT-PCR method from mRNA isolated from the cells of skunk cabbage.

In general, polymorphism is frequently recognized in the genes of eucaryotic cells. In the invention, accordingly, in addition to cDNAs represented by SEQ ID NOS: 1 and 3, those in which one or several nucleotides are added, deleted and/or replaced by (an)other nucleotide(s) in the above cDNA are included. Similarly, proteins in which one or more amino acids are added, deleted and/or replaced by (an)other amino acid(s) due to change of the above nucleotide are also included in the present invention.

In cDNA of the invention, DNA fragments (10bp or more) comprising an optimal part of the base sequences of SEQ ID NOS 1 and 3 are included. In addition, DNA fragments comprising a sense strand or anti-sense strand are also included.

The proteins of the invention, SfUCPA and SfUCPB, may be prepared respectively by a known method, for example, isolation from the spadix of skunk cabbage, preparation by chemical syntheses based on the amino acid sequence provided by the invention, or production by a recombinant DNA technique using cDNA provided by the invention. For example, when the protein is produced by a recombinant DNA technique, RNA is prepared from a vector containing cDNA of the invention by *in vitro* transcription, and this is used as a template for *in vitro* translation to yield the protein. Alternatively, the translational region of cDNA is incorporated into an appropriate expression vector according to a known method,

and th resulting recombinant vector is introduced into *Escherichia coli*, *Baccillus subtilis*, yeast, animal or plant cells. The resulting transformants can be used in expression of th proteins in a large quantity.

In the case of the proteins of the invention being produced by *in vitro* translation, the translation region of cDNA of the invention may be incorporated into a vector containing RNA polymerase promotor, and then added to an *in vitro* translation system such as a rabbit reticulocyte lysate or wheat germ extract containing an RNA polymerase corresponding to the promotor. The RNA polymerase promotor is exemplified by T7, T3, SP6, and similar promoters.

In the case of the proteins of the invention being expressed in a microorganism such as *Escherichia coli*, the translation region of cDNA is incorporated into an expression vector containing an origin replicable in microorganisms, promoter, ribosome binding site, cDNA cloning site, terminator, and the like, to construct a recombinant expression vector, which is then introduced into a host cell and incubated. In this operation, an initiation codon and a stop codon may be added to the front and tail of an optional translation region to obtain a protein fragment containing the optional region. Alternatively, the desired protein may be expressed as a fusion protein with another protein, which may be cleaved with a suitable protease to yield the desired protein. The expression vectors for *Escherichia coli* are exemplified by pUC series, pBluescript II, pET expression system, pGEX expression system, and the like.

In the case of the proteins of the invention being expressed in eucaryotic cells, the translation region of cDNA of the invention is incorporated into an expression vector for eucaryotic cells containing a promoter, splicing region, poly(A) additional site, and the like, and introduced into the eucaryotic cells. The expression vector is exemplified by pKA1, pCDM8, pSVK3, pMSG, pSVL, pBK-CMV, pBK-RSV, EVB-vector, pRS, pYES2, and the like. The eucaryotic cells,

many include mammal cultured cell such as monkey renal cell COS7, Chinese hamster ovarian cell CHO, etc., budding yeast, fission yeast, silkworm cell, Xenopus egg cell, and the like are commonly used, but not limited thereto. In order to introduce the expression vector into eucaryotic cells, a known method such as electroporation, calcium phosphate method, liposome method, DEAE dextran method, and the like can be utilized.

After expression of the proteins in procaryotic cells or eucaryotic cells according to the aforementioned method, the desired proteins are isolated and purified from the culture in the known combined procedures for separation. For example, treatment with a denaturant (e.g., urea) or surface activator, ultrasonication, digestion with enzymes, salting-out or solvent precipitation, dialysis, centrifugation, ultrafiltration, gel filtration, SDS-PAGE, isoelectric focusing, ion-exchange chromatography, hydrophobic chromatography, affinity chromatography, reverse phase chromatography, and the like are involved.

The proteins of the invention, SfUCPA and SfUCPB, also include peptide fragments (5 amino acids or more) involving the optional partial amino acid sequences of SEQ ID NOS: 2 and 4. In addition, the proteins of the invention also include fusion proteins with other optional proteins.

The following examples serve to illustrate the invention of this application specifically in more detail, but are not intended to limit the scope of the invention.

Example 1: Cloning of cDNA

The total RNA was extracted from the spadix of skunk cabbage (*Symplocarpus foetidus*) and the complete RNA was determined on 1.0% agarose gel electrophoresis (Ito et al., 1999). Using a mRNA isolation kit (Pharmacia), a

clone associated with the UCP gene family was isolated from the purified poly(A)+RNA by RT-PCR. The first strand cDNA was prepared by annealing 20 pmol of cDNA primed primer (5'-TTTTTTTTTTTTTTTTTTTTTTT-3') into poly(A)+RNA (0.1μg), followed by extension with 10 units of reverse transcriptase (New England Biolab) at 37°C for 30 minutes in 20μl of 1×RT buffer containing 10mM 1,4-dithiothreitol and 0.5mM dNTP. The composition of the reaction solution is as follows.

- 10mM Tris-HCl (pH 8.0);
- 50mM KCl;
- 1.5mM $MgCl_2$;
- 4mM dNTP;
- 0.2 unit of EX Taq polymerase (Takara); and
- 10pmol of two degenerate primers corresponding to the conserved amino acid sequence of the UCP family:

ZF1 (5'-CCIYTIGAYACIGCIAAR-3')

ZR1 (5'-ACWTTCCAISYICCIAWIC-3')

PCR cycle was carried out as follows.

(94°C: 0.5 minute; 50°C: 1 minute; 72°C: 1 minute)×35

Among the PCR products obtained in the above method, the amino acid sequence estimated from the sequence of about 0.8kb cDNA fragment had very high homology to one of the reading frame sequences of the UCP gene family. This fragment, accordingly, was cloned into T-vector (clone p2-1) and used as a probe for library screening.

cDNA (5μg) prepared from the spadix was inserted into λgt11 phage according to the known method (Sambrook et al., 1989) to construct a cDNA library. From this library, 8 clones positive to the above-described probes were isolated and sub-cloned into the pBluescript SK plasmid (Stratagene). From

these clones, clones pz8-1 and pz8-2 were btained, which respectively contained the full length SfUCPa cDNA and SfUCPb cDNA.

The insert in each clone was sequenced with an auto-sequencer ABI373A using the BcaBest sequencing kit (Takara) and T3, T7 and gene-specific primers. The sequence data were analyzed by means of the GENETYX-Homology Software System version 2.2.0 (Software Development).

cDNA of SfUCPa had the 1,525bp base sequence of SEQ ID NO: 1, and cDNA of SfUCPb had the 2,991bp base sequence of SEQ ID NO: 3. An estimated polyadenylated signal (aataaa) was found upstream of 236bp from the poly(A) sequence in cDNA of SfUCPa, while in cDNA of SfUCPb two polyadenylated sites were recognized at the positions of 1,171bp and 1,243bp. It is noteworthy that cDNA of SfUCPb has a longer 3'-untranslation region than that of SfUCPa.

cDNA of SfUCPa had an open reading frame (ORF) encoding 303 amino acids as shown in SEQ ID NO: 1, and this ORF was found to encode the protein SfUCPA of the estimated molecular weight 32.6kDa having the amino acid sequence of SEQ ID NO: 2. On the other hand, cDNA of SfUCPb had an ORF corresponding to 268 amino acids as shown in SEQ ID NO: 3, and found to encode the protein SfUCPB of the estimated molecular weight 29.0kDa.

Moreover, it was confirmed from the results of Southern blot analysis that the genome of skunk cabbage contains multiple copies of SfUCPa gene and a single copy of SfUCPb (data not shown).

Example 2: *In vitro* translation of cDNA

The plasmid clones pz8-1 and pz8-2 obtained in Example 1 were

linearized, on which a sense- or anti-sense RNA was transcribed with T7 RNA polymerase or T3 RNA polymerase according to the protocol of MAXICRIPT transcription kit (Ambion). An equal amount of RNA (4μg) was provided for in vitro translation reaction using a wheat germ extract (Promega) in the presence of ^{35}S-methionine (Amersham). The translation product was analyzed by SDS-PAGE. The gel was fixed, incubated in Amplify (Amersham), then dried, and fluorometrically analyzed.

As a result, it was confirmed that, as shown in Fig. 8, the initiation codon and the stop codon of cDNA isolated in Example 1 functioned successfully since a protein having an expected molecular weight was produced from any of cDNAs only when the sense RNA was used as a template.

Industrial Applicability

As described previously, this application provides novel thermogenic genes SfUCPa and SfUCPb as well as their gene products, i.e., thermogenic proteins SfUCPA and SfUCPB, derived from skunk cabbage (*Symplocarpus foetidus*), and cDNAs used for gene engineering mass production of these proteins. These genes and proteins allow development of low temperature-tolerant plants, development of drugs or methods for treatment of diabetes mellitus or obesity, or development of novel heat generating materials from plants.

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